ACIDIC RESIDUES ESSENTIAL FOR PROTON-ENERGIZED TRANSPORT IN THE TYPE-III SECRETION APPARATUS

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ABSTRACT

Type III secretion system in bacteria flagella functions to transfer the protein subunits that form the filament and other structures to outside the membrane. This apparatus membrane is mainly composed of FlhA, FlhB, FliO, FliP, FliQ and FliR, which form the export gate complex, while the delivery is offered by $FliH_2$-FliI complex in cytoplasm engernized by PMF. FlhA, FlhB, FliP are essential proteins for apparatus fuctioning. Further studies are performed to get more information about the roles of proteins mentioned. Mutagenesis of Flip by tryptophan shows some positions can not tolerate bulky substitutions, while mutant FlhA shows different function when substituted with differently charged residues. The results show that residues Asp 158 and Asp 208 of FlhA are important for flagellar export. Certain of the Asp 208 replacements, but not the Asp 158 replacements, could be suppressed by mutations in other residues of FlhA; thus, the requirement for an acidic residue appears most strict at position 158. The results are discussed in the framework of a transport mechanism based on proton-driven conformational changes in cytoplasmic domains of FlhA.

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CHAPTER 1

INTRODUCTION

Abstract

Bacteria have a number of different secretion pathways that enable transport of proteins, or sometimes nucleic acids, from the cytoplasm to the cell surface, the cell exterior, or into the cytoplasm of a host cell. The so-called type III secretion system (T3SS) is the most complex one. Through the action of a type III secretion system called the injectisome, many Gram-negative pathogens can inject their virulence factors into host cells. Most extracellular components of flagella are also exported by a T3SS, which is housed inside the flagellar basal body. This highly organized flagellar T3SS allows the rapid and efficient assembly of this complex multicomponent machine. The structure and mechanism of the flagellar T3SS will be reviewed in this chapter. To provide context for the discussion of type III secretion, a brief suvey of other mechanisms of bacterial secretion is also presented.

Literature review

The type III secretion system (T3SS) is an apparatus that functions in protein export in some Gram-negative bacteria. It functions in two principal contexts. One is the flagellar T3SS that is used to export extracellular components of the flagellum, which include the subunits of the rod, hook, and filament. The other setting in which type III secretion occurs is the injectisome, an apparatus that is used by many Gram-negative pathogens to inject virulence factors into host cells (1,2). Type III secretion is one of the seven known types of bacterial secrection systems (Figure 1.1), each of which is different in terms of its function, protein composition, and mechanism (3). To provide context for the discussion of type III secretion, each of these other modes of export will be briefly surveyed. The type 1 secretion system, or T1SS, is related to the ATP-binding cassette (ABC) family of transporters that is common in all three domains of life. T1SS transporters can export toxins such as proteases and lipases across both membranes of Gram-negative bacteria. The component shared with the ABC-type of transporter is an inner membrane ATPase. In addition, T1S systems contain an outer membrane, channel-forming protein (OMP) and a so-called membrane fusion protein (MFP), which spans the periplasmic space and connects the inner membrane and outer membrane. T1SS is dependent on the Sec pathway, because the channel-forming protein is exported by the Sec complex in the inner membrane. Substrates of the T1SS have an uncleaved carboxy-terminal signal sequence, which is bound to the MFP during secretion. The periplasmic MFP interacts with both the ATP-hydrolyzing component in the inner membrane and the channel-forming protein in the outer membrane, contributing to

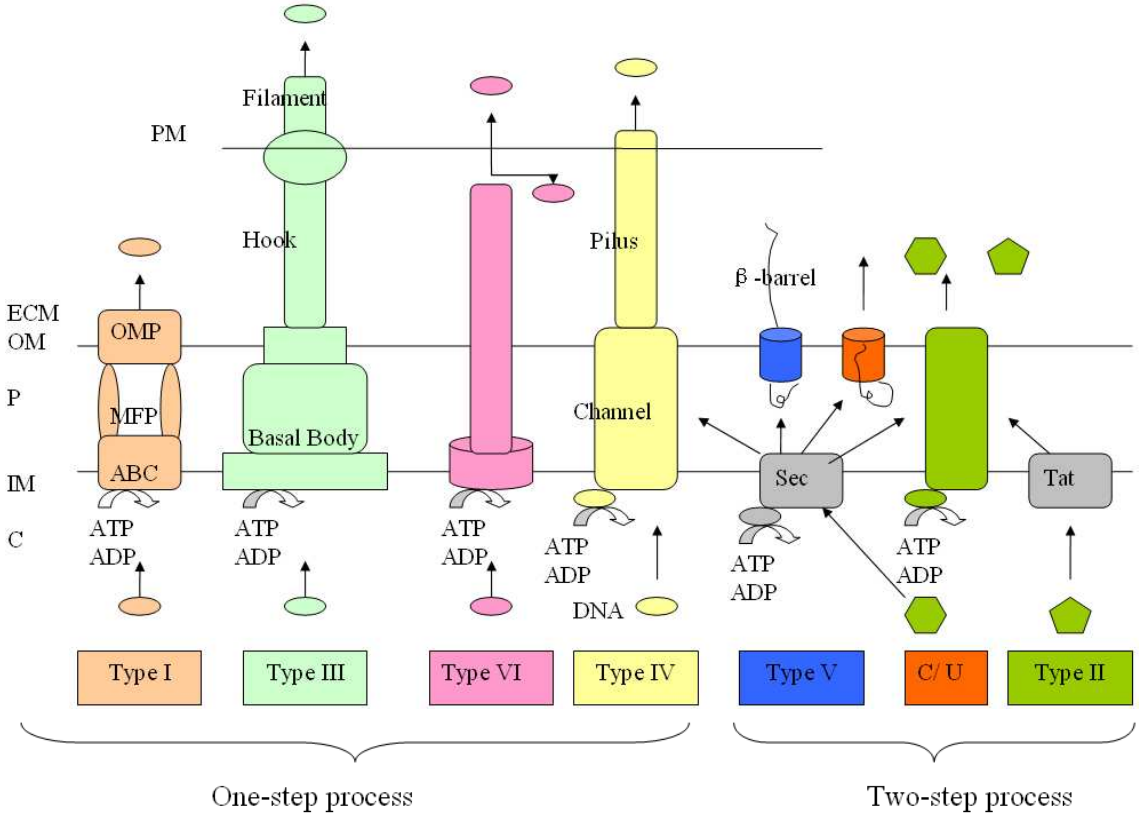

Figure 1.1. Features of some protein-secretion systems in Gram negative bacteria. PM: Host cell membrane; OM: outer membrane; P: periplasm; IM: inner membrane; C: cytoplasm.

formation of a continuous pathway for the substrate. The ATPase activity of the inner membrane component might help to release substrate (3,4).

The type 2 secretion system (T2SS) can export a variety of toxins or hydrolytic enzymes through the cell envelopes of Gram-negative bacteria. It is Sec or Tat signal dependent, because the substrate is first delivered into periplasm through the Sec or Tat complex on the inner membrane, using the Sec or Tat translocase (5). The Sec pathway only transports unstructured substrates, while Tat pathway is able to transport folded proteins (6). The following step is the secretion across the outer membrane through secretin complex encoded by a cluster of *gsp* genes, during which the N-terminal signal sequence is cleaved . Thus T2SS usually exports folded and mature proteins.

The type 4 secretion system (T4SS) is homologous to the conjugation machinery utilized by various Gram-negative and Gram-positive bacteria. It can transport not only protein but also DNA from both Gram-negative and -positive bacteria. The type 4 secretion system that has been best studied is the VirB system in *Agrobacterium tumefaciens* (7). The VirB system helps T-DNA to be delivered into plant cells. This system includes 12 proteins named VirB1 through 11 and VirD4. VirB4 and B11 have nucleotide-binding and hydrolysis activity to obtain energy and probably energize the substrate translocation through the inner membrane. VirB6, VirB7, VirB8, VirB9 and VirB10 probably compose the translocation channel in the periplasm. VirB2 is the major pilus component outside the cell, while VirB5 is a minor one (8). The molecular mechanism of type 4 export is still unclear.

The type 5 secretion system (T5SS) can export various toxins from Gram negative bacteria. It depends on the Sec system to deliver the substrate into the periplasm, similar

to T2SS. Three different families of T5SS have been reported: The autotransporter (AT) system (Type V_a or AT-1), the two-partner secretion pathway (Type V_b), and the Type V_c system or AT-2. Type 5 secretion systems are described as autotransporters, because ATP is probably not the energy source for substrate translocation. It might be the protein itself that enables completion of the transport process through a conformational change. Specifically, it is thought that once the protein has entered the periplasm, its signal sequence is cleaved, then a part of the protein termed the β-domain inserts into the outer membrane, leading to the formation of a β-barrel pore. The rest of the polypeptide (the passenger domain) is then translocated to the bacterial cell surface through the pore. The two-partner secretion pathway works in a similar way, except the passenger domain and pore forming β-domain of the two-partner secretion pathway are two separate proteins instead of a single polypeptide. The last of the three T5SS families, Type V_c system, has a very short pore forming β-domain, three copies of which are needed to form a β-barrel (9,10).

The type 6 secretion system (T6SS) has been best studied in *Rhizobium leguminosarum*, *Pseudomonas aeruginosa* and *L. pneumophila*. The secretion proteins of T6SS usually lead to disease or disorder of host cells. There are three main conserved components of the T6SS: The IcmF- / IcmH-like proteins homologous to components of the T4SS, the ClvP AAA+ ATPase, and the Hcp and VgrG (valine-glycine repeats) proteins. The ClvP AAA+ ATPase might be the energy source of T6SS. Recent research shows that T6SS has evolutionary connections with T4 bacteriophage(11-14).

The chaperone/usher (C/U) pathway is used for secretion of different virulence-related surface structures of Gram-negative bacteria. It was first found in *E*

coli. The substrate is first delivered into the periplasm through the Sec pathway, similarly to T2SS. The subsequent export through the outer membrane needs only two proteins: a periplasmic chaperone protein and channel-forming outer membrane "usher." The conserved C-terminal domain of the chaperone is first bound to the substrate. Then this complex interacts with the usher in the outer membrane. Organelles assembled by this pathway include P pili and type 1 pili of pathogenic *Escherichia coli*. These pilus consist of a thin, flexible tip fibrillum connected to a rigid, helical rod. The usher channel allows only a linear fiber of folded subunits to go through. The pilus rod is then forced to extend and twist, and finally reaches its helical conformation at the cell surface. The C/U pathway might not require external energy to deliver the pilus across outer membrane (15-20).

The type III Secretion System (T3SS) is the most complex export apparatus of the seven. Many Gram-negative pathogens inject their virulence factors into host cells using a T3SS that is sometimes termed the injectisome. Most extracellular components of flagella are also exported by the flagellar T3SS. The injectisome has a hollow needle that can allow transfer of effectors into host cells. In both the flagellum and the injectisome, protein subunit export by the T3SS is very fast. For example, the flagellar T3SS can deliver flagellin at a maximum speed of more than a thousand residues per second. T3SS has been studied in many species including *Yersinia* spp., *Salmonella* spp, *Shigella* spp, enteropathogenic and enterohemorrhagic *E. coli, Pseudomonas aeruginosa, Chlamydia trachomatis* and *Chlamydia pneumoniae, Bordetella bronchiseptica, Bordetella pertussis,* and *Burkholderia pseudomallei*. (21,22).

The T3SS of injectisomes enables the assembly of an external appendage that helps the bacteria to dock on the surface of host cells, then to deliver toxins into those cells. The virulence factors usually have enzymatic activities. In some plant pathogenic bacteria, for example, the effector proteins are related to cysteine protease or protein tyrosine phosphatase activity (23). The effect is to down-regulate host defenses. In animal-pathogenic bacteria, the effector proteins target cellular signaling molecules such as small GTP-binding proteins, mitogen-activated protein kinases (MAPKs), IκB-α and phosphoinositides (24). Their action is to down-regulate pro-inflammatory responses.

In the flagellum, T3SS delivers the components that form the extracellular structures. The exported subunits are of three kinds: Rod-type (FliE, FlgB, FlgC, FlgF, FlgG), hook-type (FlgD, FlgE, FliK), and filament type (FlgK, FlgL, FliD, FliC, FlgM). Their order of export parallels the assembly order: first rod, then hook, and lastly the filament (25-27).

In both the flagellum and the injectisome, type III secretion is mostly powered by proton motive force (pmf) (28,29). The first indication of this came from the observation that export by the T3SS in *Yersinia enterocolitica* was prevented by treatment with CCCP (30). Subsequent work in the flagellar system showed that pmf is essential, but ATP hydrolysis dispensable, for export. Both the injectisome and flagellar systems contain ATPases that were thought critical for export; in the flagellar system this component is FliI. FliI binds to, and is regulated by, FliH. FliI and FliH function together, along with a third protein called FliJ, to deliver substrate molecules to the inner mouth of the export apparatus. ATP is thought to facilitate the release of the FliH-FliI complex from the gate and from the substrate (28,31). However, details of how the cargo is delivered, and to

where, are still unknown. Most importantly, how the cargo is actively moved across the membrane, using the pmf, is still unclear.

Composition and structure of the T3SS apparatus

In both the flagellum and injectisome, the T3SS consists of some cytoplasmic proteins, a large assembly in the membrane, and exterior components that form a relatively long, slender appendage. Both contain a core set of proteins that show clear homology. The proteins shared in common between the flagellum and injectisome are listed in Table 1.1. A sketch of the flagellar T3SS, in context with the flagellar structure is displayed in Figure 1.2.

Exterior components

In place of the flagellar hook and filament, the injectisome has a so-called needle complex (NC). The flagellar filament functions as a propeller and is accordingly relatively large, measuring about 10 to 15 nm in length, and 120-240 nm in diameter. The NC of the injectisome is somewhat smaller, measuring about 60 nm in length and 7 nm in diameter. The filament has a specialized cap structure which ensures that flagellin subunits are polymerized onto the end of the structure rather than escaping into the medium. The NC is a passage for the secretion proteins to be injected into host cells, and is also capped by a specialized structure, which in this case functions to dock onto the host cell and form a translocation pore to allow delivery of the virulence effectors into the host-cell cytoplasm (2,11,25). Proteins that form these structures are listed in Table 1.1. In *Salmonella*, the exported virulence factors, which are not listed, are encoded by the *Salmonella* pathegenity island 1 (SPI-1).

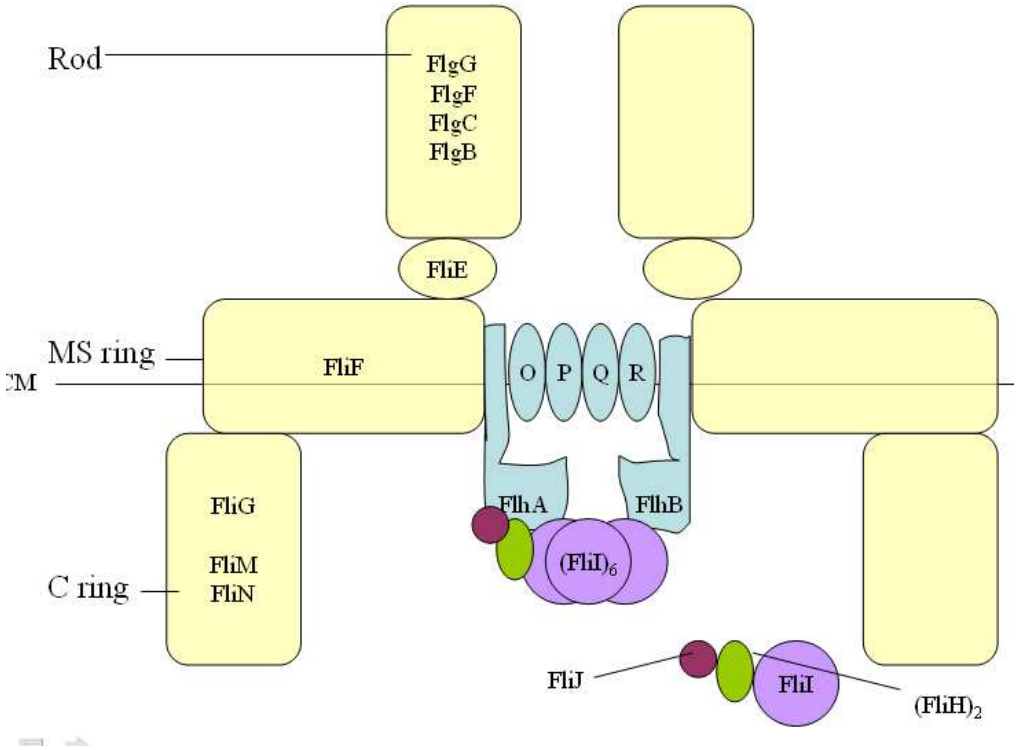

Fig.1.2. Overall organization of the flagellar T3SS. Membrane-bound components are shown in single copies but are likely to be present in multiple (but presently unknown number of) copies.

Table. 1.1. Conserved proteins of the flagellum and injectisome of *S. enterica*.

Flagellum (*S.enterica*)	Injectisome	Location and function
FliF	PrgH/PrgK	Inner-membrane ring
FliI	InvC	ATPase
FliH	YscL	ATPase regulator
FliJ	--	Positioned in FliI hexamer
FliGMN	PrgJ/SpaO	C ring (flagellum)
FliP	SpaP	T3SS inner membrane protein
FliQ	SpaQ	T3SS inner membrane protein
FliR	SpaR	T3SS inner membrane protein
FlhA	InvA	T3SS inner membrane protein
FlhB	SpaS	T3SS inner membrane protein
--	InvG	Outer membrane ring
FlgE?	PrgI	Extracellular needle
--	SipBC	Translocation pore
FliC?	SseB	Needle extension
FliK	InvJ	Hook/needle length regulator

Membrane components

Both the flagellum and injectisome have ring-like structures within the cytoplasmic membrane. In the flagellum, this feature is called the MS-ring, and is larger than the corresponding feature of the injectisome. Both the flagellum and injectisome have outer rings that span the peptidoglycan and outer membrane; in the flagellum these are termed the L and P rings. Flagella contain a large feature in the cytoplasm called the C ring. The C-ring is also called the switch complex, because it functions to regulate reverals between clockwise and counter-clockwise rotation of the flagellum. The injectisome has a smaller feature in place of the C-ring, presumably reflecting its more-narrow function, which does not involve direction switching (1,2).

The cytoplasmic ring structures house several other membrane components, which are the most important in the sense that they form the actual export pathway. They are presently the most mysterious components as well, as we know very little about their structure, number, or organization within the appartus. These components include FliP, FliQ, FliR, FliO, FlhA, and FlhB (32-34). An additional components called FliO occurs in most flagella, but has been found nonessential under some circumstances (35), and is not found in injectisomes. Membrane topologies are fairly well established for most of these components, though some uncertainties remain, as will be discussed in Chapter 2.

FliP and FliR have been identified in purified basal bodies, and FliR has been located at the cytoplasmic face of the MS ring by immuno-electron microscopy (36). FliO is reported to maintain FliP stability through transmembrane domain interaction, but, as noted, FliO has been found nonessential for flagellar assembly under some circumstances (specifically, when FliP is moderately overexpressed) (32,35). FlhA and FlhB have large

cytoplasmic domains, which project into the cytoplasmic region enclosed by the C ring. They are believed to form the "gate platform," to which substrate is delivered, in complex with the cytoplasmic components FliH, FliI, and FliJ (using names from the flagellar system) (37-39). It has been suggested that FlhA or FlhB might contribute to the proton translocation pathway of the export apparatus, but there is no evidence for this.

In both the flagellum and the injectisome, a transition in substrate specificity appears to allow the apparatus to shift from exporting early substrates (which include the rod and hook subunits in the case of the flagellum) to so-called late substrates (subunits of the filament, and associated proteins). FlhB, and the corresponding protein of the injectisome, are thought to play a central role in this specificity switch. These proteins undergo an appropriately delayed autocleavage process that is one of the determinants of the specificity change. Another component involved in the switch is FliK, which is believed to function as a ruler to measure the hook length and then interact with FlhB at the appropriate time to trigger the switch (40-42).

Cytoplasmic components

The cytoplasmic components of the T3SS, already mentioned briefly, are FliH, FliI, and FliJ. Although these components were shown to be not essential for the assembly of flagella, export is clearly less efficient without them, and they are found universally in all flagellar and injectisome systems (37,43).

FliI is an ATPase and was thought, until relatively recently, to provide the energy for substrate transport. It is homologous to the alpha/beta subunits of ATP synthase, and like those proteins, can associate to form hexamers in the presence of ATP and

phospholipids. FliH forms dimers, and interacts with and inhibits the ATP hydrolysis activity of FliI. FliH shows weak homology to the β subunit of ATP synthase. It also interacts with the protein FliN, which is a component of the C-ring, and could thus function to target FliH/I/J/substrate complexes to the vicinity of the export apparatus. The FliH/I/J complexes, and hydrolysis of ATP, are thought to be important for efficient delivery of the cargo. Following hydrolysis of ATP, the $FliH_2FliI$ complex dissociates from the gate and from the export substrate protein. The subsequent translocation of the substrates is driven by PMF (28,43,44).

The function of FliJ is presently the least clear. It has been suggested to function as a general chaperone, which interacts with FliH, FliI export substrate, and the cytoplasmic domains of FlhA and FlhB. It has also been suggested to fulfill the role of "chaperone of chaperones," interacting with the FliS, FliT, and FlgN proteins that function as chaperones for particular flagellar proteins. It displays weak homology to the gamma subunit of ATP synthase, and has been shown to occupy an analogous position, within the center of the FliI hexamer (40,45-47).

Major questions remaining

While it is now clear that the energy for substrate translocation across the membrane is provided by the protonmotive force, the molecular mechanisms of substrate movement and coupling to the proton gradient remain unknown. It is also not clear how the cargo is delivered, nor which protein or motif takes part in the delivery or accepts the cargo. My research has focused mainly on the question of what proteins, and what groups, might be involved in coupling to the proton gradient. Specifically, I sought to

identify conserved, protonatable groups that might function in proton-transfer processes within the export apparatus. Candidate proteins included FliP, FliQ, FliR, FlhA and FlhB, as these constitute the conserved core of the membrane-bound part of the T3SS. Patterns of sequence conservation were used as a further guide, to target particular residues for mutageness. The results identify candidate positions that are likely to function in coupling the proton gradient to substrate transport, and provide a foundation for probing the mechanism in fuller molecular detail.

CHAPTER 2

ACIDIC RESIDUES ESSENTIAL FOR PROTON-ENERGIZED TRANSPORT IN THE TYPE-III SECRETION APPARATUS

Abstract

The basal body of the bacterial flagellum contains a specialized secretion apparatus that functions to export protein subunits that form the rod, hook, and filament structures to their sites of installation on the outside of the cell. A closely related apparatus is present in the 'injectisome' of Gram-negative pathogens, where it functions to export virulence factors into host cells. This class of protein export is termed type-III secretion. Export by this mechanism can be notably fast; during growth of the flagellar filament, for example, the ~55-Kd flagellin subunits are secreted at a rate of several per second. This rapid secretion is made possible by the use of two energy sources: Protein subunits destined for export are delivered to the inner face of the apparatus in a process energized by ATP, and then are transported across the membrane using energy derived from the proton gradient. The molecular mechanism of the proton-energized transport process is unknown. In the present study, conserved acidic and basic residues of the membrane-bound components of the apparatus were mutated systematically to identify potential sites of proton interaction. The results show that residues Asp 158 and Asp 208 of FlhA are important for flagellar export. Certain of the Asp 208 replacements, but not

the Asp 158 replacements, could be suppressed by mutations in other residues of FlhA; thus, the requirement for an acidic residue appears most strict at position 158. The results are discussed in the framework of a transport mechanism based on proton-driven conformational changes in cytoplasmic domains of FlhA.

Introduction

Flagellar assembly begins with structures in the vicinity of the cytoplasmic membrane and proceeds through a series of steps that add the exterior structures in a proximal-to-distal sequence (48,49). Assembly of the rod, hook, and filament requires the action of the flagellar type III secretion (T3S) apparatus, which delivers protein subunits to their sites of installation by way of an axial channel through the structure (50,51). The flagellar export system includes an ATPase, called FliI (43), which was at first believed to provide the energy for export. More recent work has shown that the translocation process does not actually require ATP but is energized by the membrane proton gradient (29,30,52,53). The T3S apparatus is thus a proton-energized protein pump. Export by the T3S apparatus can be very fast; in the early stages of flagellar assembly several fairly large (~55Kd) flagellin subunits are transported per second (54).

The membrane part of the flagellar export apparatus is formed from the proteins FlhA, FlhB, FliP, FliQ and FliR (33,34). FliO also contributes to optimal export function but recent studies indicate that *fliO*-deletion strains retain some ability to assemble flagella and can be rescued either by over-expression or mutation of FliP (32,35). The export apparatus is believed to be housed within the MS-ring (49,55,56), a large membrane-associated feature of the basal body formed from about 25 copies of the

protein FliF (57). A recent FRET-based study suggested that some components might lie in the membrane adjacent to the basal body (58), but the functional significance of such peripherally-located subunits remains to be established. FliH, FliI and FliJ are soluble (i.e., nonmembrane) proteins that function in the cytoplasm to deliver cargo to the base of the apparatus (43,59-62). Export of certain cargo subunits is further assisted by the substrate-specific chaperones FlgN, FliS, and FliT (45,63-66). An understanding of flagellar export is of considerable medical relevance, as the flagellar T3S apparatus is closely related to the 'injectisome' that functions in Gram-negative pathogens to transport virulence factors into the cells of the host (66-68).

The membrane components of the flagellar export apparatus vary considerably in size and in the number of trans-membrane (TM) segments (Figure 1.2). It is not known how many subunits of each protein are present in the apparatus, but the membrane patch enclosed by the MS-ring should be large enough to accommodate multiple copies of each (49). FlhA and FlhB contain large carboxyl-terminal domains on the cytoplasmic side of the membrane that engage the FliH/FliI/FliJ cargo-delivery complex (33,39,41,61,69-74). FlhB regulates a change in substrate specificity that occurs on completion of the hook, enabling the export of late-class proteins that form the flagellar filament (41,42,74). FliP and FliR have been found in purified basal bodies (36), and intergenic suppression studies identified an interaction between FlhA and FliF (38), in support of the hypothesized location of the apparatus within the MS-ring. Hara *et al*. (75) recently reported additional instances of intergenic suppression that provide further evidence of associations among the FlhA, FlhB, and FliR proteins (75). FlhB and FliR are likely to occur in equal numbers and in proximity, because some *Clostridia* contain a naturally

occurring *fliR-flhB* genetic fusion (76), and an engineered FliR-FliB fusion was shown to retain function in *Salmonella* (76,77). A recent analysis of the functional contributions of individual membrane components gave evidence that FliP has a major role in forming the conduit for substrate (35).

Although the membrane proteins of the T3S apparatus were identified several years ago, the details of their organization and the molecular mechanism of substrate translocation remain unclear. In contrast to the soluble components FliH, FliI, and FliJ, which are evolutionarily related to components of the ATP synthase (46,47), the membrane components do not appear to be related to other, better-studied transport systems. Some generalizations regarding the mechanism may apply nevertheless; we might, for example, expect the T3S apparatus to contain critical titratable groups such as have been identified in other proton-energized systems including the ATP synthase (78-80), the *E. coli* lactose transporter (81), and the flagellar rotary motor (82). The membrane proteins of the flagellar export apparatus contain several conserved acidic and basic residues, in both the TM segments and adjoining regions, which might function to bind the energizing protons. Hara *et al*. (75) recently undertook a mutational analysis of eight conserved protonatable residues in the TM segments of FlhA and found that FlhA residue Asp 208 is important for function. In the present study, we sought to identify functionally important proton-binding residues of the T3S apparatus by systematic mutagenesis of the conserved acidic and basic residues in each of the membrane components. We confirm that FlhA residue Asp 208 is important for normal function, but find that it is dispensable under some circumstances. FlhA residue Asp 158, which lies in a strongly conserved cytoplasmic domain, appears most critical for function of the T3S

apparatus. Proton-binding residues in the other membrane components (FlhB, FliP, FliQ, and FliR) are either dispensable for function or have properties that suggest their involvement in processes other than proton coupling. The results are discussed in the framework of a simple transport mechanism based on proton-regulated conformational changes in cytoplasmic domains of FlhA.

Materials and methods

Strains, plasmids and mutagenesis

Experiments used strains of *Salmonella enterica* (serovar *Typhimurium*) listed in Table 2.1. The *fliP, fliQ, fliR , flhA*, and *flhB* genes were cloned into the salicylate-inducible expression vector pKG116, a gift from J. S. Parkinson (University of Utah), using *NdeI* and *KpnI* sites. Mutations were made using the Altered Sites (Promega) or QuikChange (Stratagene) methods, and were confirmed by sequencing.

Function of mutated proteins

For assays of function, salicylate-inducible plasmids encoding the mutated proteins were transformed into corresponding deletion strains, and rates of migration in soft-agar tryptone plates (0.27% Bacto-agar, 10 g/L tryptone, 5 g/L NaCl) were measured as described previously (83), with induction by salicylate at the levels indicated in the figures and tables. For tests of dominance, the mutant plasmids were introduced into the wild-type strain LT2, and rates of migration in soft agar were measured relative to that of the wild-type strain expressing the wild-type protein from the plasmid. Function of some FlhB mutants was tested in the presence of over-expressed wild-type FlhA. These

Table 2.1. Strains and plasmids

Strain	Relevant genotype or property	Source or reference
LT2	Wild type *Salmonella serovar typhimurium*	K. T. Hughes
TH12642	*flhA* null strain	K. T. Hughes
TH12644	*flhB* null strain	K. T. Hughes
TH10549	*fliP* null strain	K. T. Hughes
TH10550	*fliQ* null strain	K. T. Hughes
TH10551	*fliR* null strain	K. T. Hughes
Plasmids		
pKG116	Salicylate-inducible vector; Cm^r	J. S. Parkinson
pMS10	*fliP* in pKG116	This study
pMS11	*fliQ* in pKG116	This study
pMS12	*fliR* in pKG116	This study
pMS122	*flhA* in pKG116	This study
pMS123	*flhB* in pKG116	This study
pMS152	*His-flhA* in pTRC99A	This study

experiments used a compatible second plasmid encoding His-tagged FlhA, which was shown to function as well as wild-type FlhA in a complementation test using the Δ*flhA* strain.

Secretion assay

FliC-secretion assays were performed essentially as described by Komoriya *et al*. (84). One-mL cultures were grown in LB-broth overnight at 37^{o}C, then diluted 100-fold into 5 mL LB and grown at 37^{o}C to an OD_{600} between 0.5 and 0.7. Cells were pelleted (18, 500 x *g*, 15 min), and a 1-mL portion of the supernatant was transferred to a 1.5-mL microfuge tube, mixed with 0.3 mL pre-chilled 25% trichloroacetic acid (final concentration 6%), chilled on ice for 15 min, and centrifuged at 10, 000 x *g* for 10 min. Pellets were washed twice to remove TCA, by re-suspension in 0.3 mL acetone and re-pelleting. The washed pellets were dissolved in SDS sample buffer and analyzed by SDS-PAGE. Proteins bands were visualized on immunoblots using anti-FliC antibody.

Membrane topology

Trans-membrane segments were predicted using the web-based tools MEMSAT-SVM (85), HMMTOP (86), SOSUI (87), TMHMM (88), TMPRED (89), and TopPred (90). For experimental examination of FliQ and FliR topology, in-frame C-terminal fusions of '*lacZ* and '*phoA* to *fliQ*, *fliR*, and *fliO* were constructed by insertion of the *Bam*HI fragment from Tn*lacZ*/in and Tn*phoA*/in respectively (containing '*lacZ cat* or '*phoA cat*) (91). (FliO has been shown to have its C-terminus in the cytoplasm (32), and was included here as an additional control.) The fusion proteins were expressed in

strain CC191 (Δ*lac*/Δ*phoA*) and activity was assayed on plates containing either 80 μg/mL X-Pho (5-bromo-4-chloro-3-indolylphosphat- p- toluidine) or 80 μg/mL X-Gal (5-bromo-4-chloro-3-indolyl-13- D- galactopyranoside), incubated overnight at 37° C.

Suppressors of Asp-208 mutations

Cells of the *flhA*-deletion strain expressing FlhA proteins with mutations in residues Asp 158 or Asp 208 were inoculated in streaks onto a soft-agar plate, and plates were incubated at 32° C to allow the outgrowth of motile variants. The Asp-158 mutants failed to produce motile variants after 36-48 hours, but some of the Asp-208 mutants displayed outgrowth within 24-36 hours, usually in multiple locations. Cells from the leading edges were colony purified and cultured to isolate the *flhA*-encoding plasmid for re-transformation into the *flhA*-deletion strain. Plasmids conferring motility were sequenced and were found in some cases to retain the original Asp-208 allele, together with a 2nd mutation in *flhA*. The double mutants were re-constructed to confirm the motility phenotype, and the suppressing mutations were also combined with each of the other Asp-208 alleles to examine allele specificity.

Results

Mutation of conserved proton-binding residues of the T3S apparatus

Sequence-based topology-prediction methods (86-90) indicate four probable trans-membrane (TM) segments in FliP, two in FliQ, six in FliR, four in FlhB, and seven or eight in FlhA (Table 2.2). A consensus view of the topologies is displayed in Figure 2.1. In a recent study of the membrane proteins in the *Xanthomonas* injectisome, reporter

TABLE 2.2. Comparison of transmembrane segment predictions for proteins of the flagellar export apparatus

	Prediction Method					
	MEMSATSVM	**HMMTOP**	**SOSUI**	**TMHMM**	**TMpred**	**TopPRED**
FlhA TM segment						
1	23-40	22-41	19-41	21-43	21-44	21-41
2	44-64	46-64	44-66	47-64	46-65	45-65
3	74-89	73-97	72-94	76-98	68-93	72-92
4	118-141	118-142	122-144	118-140	123-140	122-142
5	210-229	210-229	208-230	213-235	209-229	209-229
6	245-260	250-269	248-270	250-272	244-266	251-271
7	291-307	292-316	298-320	293-315	310-327	289-309
8	311-326					
8a						473-493
FlhB TM segment						
1	31-49	33-50	33-55	33-50	33-51	31-51
(2)			72-94			
2	81-111	91-115	99-121	93-115	96-117	88-108
3	144-174	146-162	144-166	146-168	149-168	144-164
4	181-211	189-213	185-207	189-211	189-207	187-207
(5)						331-351
FliP TM segment						
1	43-73	47-71	48-70	47-69	45-64	42-62
2	89-104	88-105	87-109	88-105	88-105	86-106
3	182-212	186-210	184-206	185-207	193-213	189-209
4	216-236	215-239	213-235	214-236	223-241	212-232

(Table 2.2 cont.)

	Prediction Method					
	MEMSATSVM	**HMMTOP**	**SOSUI**	**TMHMM**	**TMpred**	**TopPRED**
FliQ TM segment						
1	13-41	17-38	17-39	20-42	16-38	19-39
2	51-78	55-74	56-78	52-74	55-74	47-67
FliR TM segment						
1	9-32	9-33	12-34	15-34	13-30	15-35
2	42-57	44-60	43-65	41-58	44-61	41-61
3	70-100	65-88	70-92	73-95	71-94	65-85
(4)					98-116	
4	126-155	128-151	132-154	132-154	122-151	121-141
5	178-207	178-202	172-194	179-201	175-193	184-204
6	211-241	213-237	224-246	214-236	213-237	211-231

fusions to a tandem LacZ-PhoA construct suggested the presence of only single TM segments in the FliR-homolog HrcS and the FliQ-homolog HrcT, in sharp contrast to all of the sequence-based predictions. We have adopted the FliQ and FliR topologies shown in Figure 2.1, as these are supported by basic considerations of hydrophobicity (the presence of multiple strongly-hydrophobic segments in both proteins) and also by experiments using the more-conventional individual fusions to LacZ and PhoA, described below. Sequence alignments of the FliP, FliQ, FliR, FlhB, and FlhA proteins from about 50 phylogenetically diverse species were used to identify conserved acidic and basic residues that might function in proton binding. These were individually replaced with alanine, by mutagenesis of the genes cloned into salicylate-inducible plasmids. Mutant plasmids were transformed into the corresponding deletion-mutant strains, and function was assayed on soft-agar motility plates.

In FliP, four acidic residues and five basic residues are either invariant or strongly conserved. Alanine replacements at most of these positions had only minor effects on FliP function as assayed in motility plates (Table 2.3). Function was decreased substantially, though still not eliminated, by mutation of FliP-residues Glu 178 (in the periplasmic domain near the beginning of TM-3) or Lys 222 (in TM-4). As we noted in a previous study (35), Asp 197 of FliP is critical for function; several Asp197 replacements eliminated motility in the soft-agar motility assay and prevented export of FliC (flagellin) (reference (35) and Table 2.3). Results in the previous study indicated a role for FliP in forming the trans-membrane conduit for substrate. Accordingly, we suggest that Asp 197 functions in forming the substrate conduit rather than in coupling to proton flow, though the formal possibility remains that it might contribute to both functions.

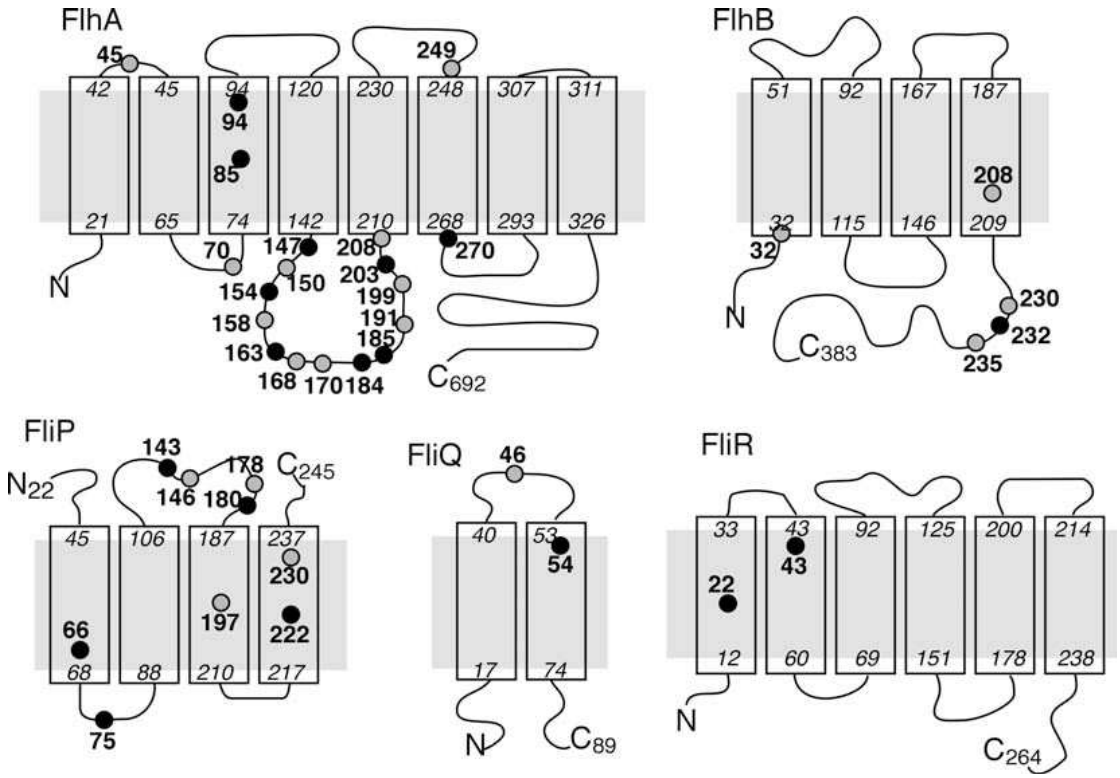

Figure. 2.1. Consensus topology predictions for the membrane components of the flagellar export apparatus. Approximate endpoints of the TM segments are indicated, using numbering for the *Salmonella* proteins. Positions of conserved acidic (gray circles) and basic residues (black circles) are indicated. All methods predict two TM segments for FliQ, and results in the present study (Figure 2.5) indicate a cytoplasmic location for the C-terminus. The eight-segment topology for FlhA was predicted by only one of the methods, but the other methods indicated a fairly long TM-7 segment with fairly widely varying endpoints that is consistent with the presence of two short TM segments. The eight-TM topology is in accord with the known cytoplasmic location of the C-terminal domain.

Table 2.3. Effects of mutations in FlhA, FlhB, FliP, FliQ and FliR

Mutation	Relative Swarm Rate[a]	Dominance[b]
FliP		
D146A	0.8	
E178A	0.2	1.3
D197A	0.0	1.0
D197N	0.0	1.0
D197P	0.0	1
D197G	0.0	1
D197E	1.0	
D230A	1.4	
R66A	0.5	1.1
R75A	0.6	1.1
R143A	0.8	
K180A	1.0	
K222A	0.2	1.0
FliQ		
E46A	0.0	1.2
K54A	0.0	1.2
G32A	0.4	
Q39A	0.5	
T42A	0.5	
Q43A	0.4	
T48A	0.5	
FliR		
R22A	0.9	
K43A	0.6	
FlhB		
E32A	1.0	
D208A[c]	0.1	0.6
D208N	0.1	0.6
D208W	0.0	1.0
D208G	0.1	1.0
D208S	0.2	1.1
D208E	0.5	
D208H[c]	0.2	0.8
E230A	0.3	
K232A	0.4	
E235A	0.3	

(Table 2.3 cont.)

Mutation	Relative Swarm Rate[a]	Dominance[b]
FlhA		
D45A	0.7	
D70A	0.2	1.0
E150A	1.4	
D158A	0.0	0.2
D158N	0.0	0.7
D158Q	0.0	0.0
D158S	0.0	0.1
D158H	0.0	0.3
D158P	0.0	0.1
D158E	1.0	
D168A	0.3	0.8
D170A	0.0	1.1
E191A	1.2	
D199A	1.1	
D208A	0.0	0.7
D208N	0.0	0.9
D208Q	0.0	0.1
D208S	0.0	0.7
D208E	0.0	0.4
D208H	0.0	0.1
D249A	0.0	0.7
R85A	1.3	
R94A	0.0	0.1
R147A	0.6	
R154A	0.6	
K163A	1.4	
R184A	0.8	
R185A	0.6	
K203A	1	0.6
R270A	1.4	

[a] Swarming rate of strains with chromosomal-deletion strains complemented with the corresponding plasmid-borne genes, induced with 2.5 μM Na-salicylate. Rates are relative to control strains expressing wild-type protein from the plasmid.

[b] Swarming rates of wild-type (LT2) cells expressing the mutant proteins from plasmids, with induction by 2.5 μM Na-salicylate, relative to controls expressing the wild-type protein from the plasmids (1.0 = recessive; 0.0 = fully dominant).

[c] Swarming in these FlhB mutants was restored to nearly wild-type levels upon over-expression of FlhA.

FliQ contains a single conserved acidic residue (Glu 46) that is located in a short periplasmic segment, and a single conserved basic residue (Lys 54) near the periplasmic end of TM-2 (Figure 2.1). Motility was eliminated by alanine replacements at either position (Table 2.3). The E46A and K54A mutant proteins were expressed in wild-type cells to assess dominance. If nonfunctioning mutant proteins are able to replace the wild-type protein in the export apparatus, they would be expected to inhibit motility when expressed in wild-type cells, as is observed, for example, with MotB proteins carrying replacements of Asp 32 (82). The E46A and K54A FliQ variants did not affect motility of the wild type (Table 2.3). This finding argues against a critical proton-conducting function and is more consistent with a role in stabilizing the protein or facilitating its incorporation into the apparatus. In addition to the conserved Glu and Lys residues, FliQ has a conserved Gly, and several highly conserved polar residues (mostly Gln) that would presumably not bind protons directly but might contribute polar groups to a proton-conducting channel. Alanine replacements of these residues caused reductions in motility, but none were critical (Table 2.3).

FliR contains one invariant basic residue, located in TM-1 (Arg 22). An alanine replacement at this position had only a minor effect on motility. Basic character is conserved at one other position in FliR, in TM-2 (Lys 43 in the protein of *Salmonella;* either Lys or Arg in other species). Lys 43 also proved nonessential (Table 2.3). FliR has no invariant acidic residues; an Asp residue occurs at fairly high frequency in one of the periplasmic segments (residue 115 in *Salmonella* FliR), but was also found to be tolerant of Ala replacement (data not shown).

FlhB contains one invariant basic residue (Lys 232), three invariant acidic residues (Asp 208, Glu 230, and Glu 235), and one moderately conserved acidic residue (Glu 32). Motility was not affected by the Ala replacement of Glu 32. Alanine replacements of Glu 230, Glu 235, and Lys 232 caused some reduction in motility but none were indispensable. The replacement of FlhB residue Asp-208, however, caused a nearly complete loss of motility, and the D208A protein also inhibited motility significantly when expressed in wild-type cells (Table 2.3). Other replacements of FlhB-residue Asp 208 were tested to examine the side-chain requirements at this position. Glu supported motility at about 50% of the wild-type rate, whereas function was eliminated by replacements with Asn, Gly, or Trp. A Ser replacement supported function at about 20% of wild-type, even though lacking a readily protonatable side-chain. Further, the FlhB-D208A mutant was substantially rescued by overexpression of FlhA in the cells (Table 2.2 and Table 2.3). Thus, while Asp 208 contributes to the optimal function of the export apparatus it does not appear to carry out a critical proton-coupling function.

FlhA contains the largest number of conserved proton-binding residues, nine basic and ten acidic (Figure 2.1). Two of the conserved basic residues (Arg 85 and Arg 94) are predicted to lie within TM segments, and most of the others are found in the very highly conserved cytoplasmic domain between TM-4 and TM-5 (Figure 2.1). Alanine replacements at several of these positions were tolerated, allowing motility similar to wild-type (Table 2.3). Replacements of three of the basic residues (Arg 147, Arg 154, or Arg 185) caused partial motility reductions, and replacements of Arg 94 or Lys 203 eliminated motility completely. Both the R94A and K203A proteins exerted moderate dominant-negative effects, decreasing the motility of wild-type cells to about half of

normal (Table 2.3) also reported a complete motility defect in the R94A mutant (19), but observed only a partial defect in the K203A mutant.

Among the conserved acidic residues of FlhA, four (Asp 45, Glu 150, Glu 191, and Asp 199) could be replaced by alanine with comparatively little effect. Motility was decreased by replacements of Asp 70 or Asp 168, and was eliminated by replacements of Asp 158, Asp 170, Asp 208, or Asp 249. In the dominance test, the D170A protein had no inhibitory effect and the D249A protein caused only a minor motility reduction. The D158A and D208A proteins displayed stronger dominance, decreasing motility of the wild type to about one-fourth of normal (Table 2.3). Asp 208 is the residue highlighted in the study of Hara *et al*. (75), and lies near the inner (cytoplasmic) end of TM-5. Asp 158 is located in the conserved cytoplasmic domain between TM-4 and TM-5 (Figure 2.1).

Additional replacements of Asp 158 and Asp 208 were tested to characterize the functional-group requirements at these positions. At position 158, motility was eliminated upon replacement of the native Asp with Asn, Gln, Ser, His, or Pro (Table 2.3 and Figure 2.2). Although Glu does not appear to occur naturally at position 158, a D158E mutant exhibited motility comparable to the wild type (Table 2.3 and Figure 2.2). The nonfunctional Asp-158 variants inhibited motility to various degrees when expressed in wild-type cells, with the Gln replacement having the strongest effect (Table 2.3 and Figure 2.2). At position 208, function was prevented by all replacements tested (Asn, Gln, Ser, His, and Glu; Table 2.3 and Figure 2.2). The position-208 mutations were also dominant to various degrees, but typically less so than the Asp-158 mutations (Table 2.3 and Figure 2.2).

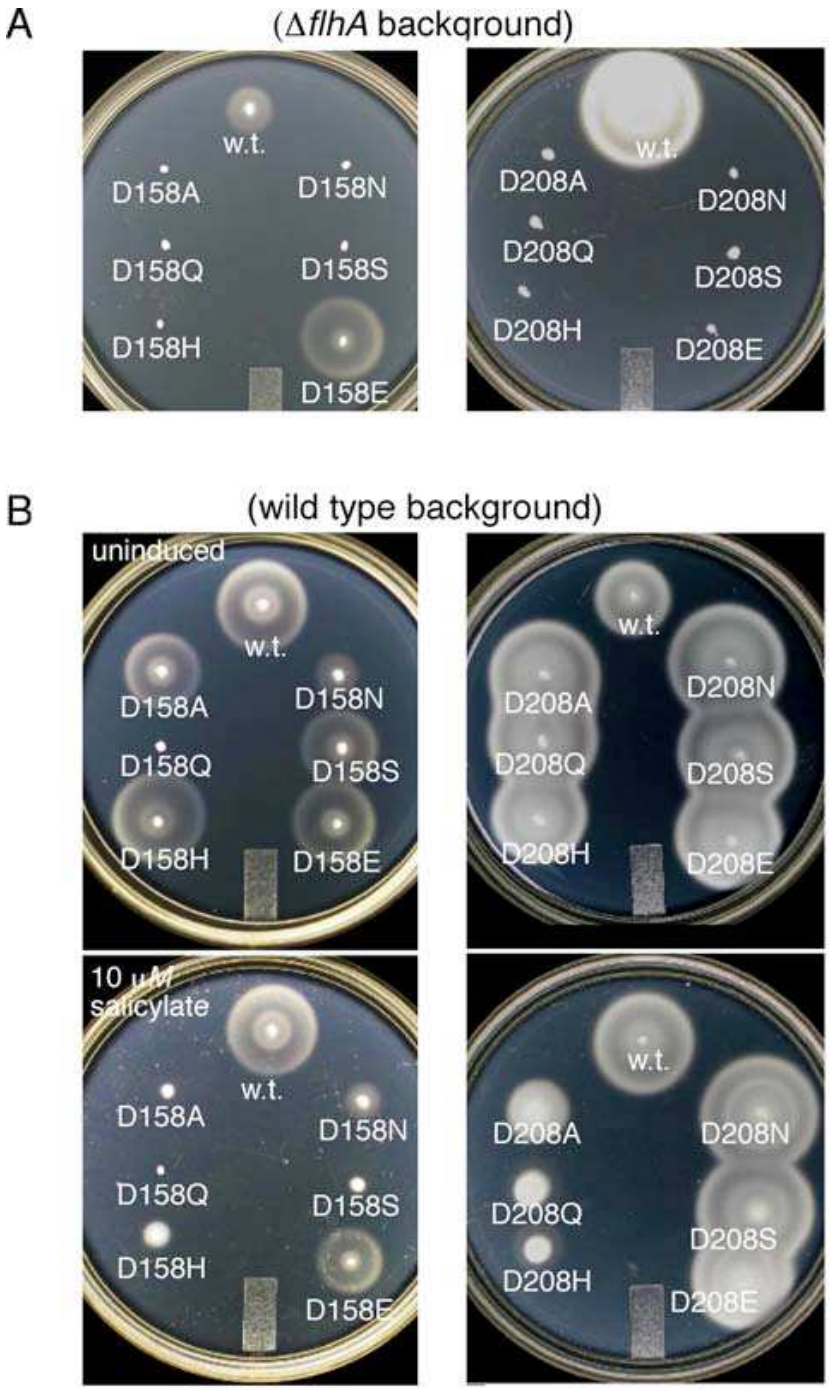

Figure 2.2. Phenotypes of FlhA mutations. A) Effects of replacements of Asp158 (left) or Asp208 (right) on motility as assayed on soft-agar plates. FlhA proteins were expressed in the Δ*flhA* strain from plasmids based on pKG116 (Table1), induced with 2.5 μM salicylate. Plates were incubated at 32° C for 6 h. B) Dominant-negative effects of the mutant FlhA proteins expressed from plasmids in the wild-type strain LT2 at either a low level (top; no added salicylate) or high level (bottom; 10 μM salicylate). Plates were incubated at 32° C for 6 h.

Intragenic suppression of Asp-208 mutations

To determine whether additional mutations might rescue the motility defects in the Asp- 158 and Asp- 208 mutants, cells of the Δ*flhA* strain expressing the Asp-158 and Asp-208 FlhA variants were inoculated in streaks on soft agar, and the plates were monitored for the appearance of outward-migrating cells. None of the Asp-158 mutants produced motile variants in several attempts by this method. Some Asp-208 mutants, however, gave rise to motile variants after 24-36 hours (Figure 2.3). Motile cells derived from the D208S and D208E mutants were isolated, and their plasmids were purified and re-transformed into the Δ*flhA* strain. In cases where the plasmid conferred motility on the Δ*flhA* strain, the plasmids were sequenced, and in two instances were found to retain the original mutation together with a second mutation in *flhA*. Double mutants isolated in this way were D208S/R85H and D208E/G223V. The suppressing mutations R85H and G223V were then paired with each of the other Asp-208 replacements, and motility was re-tested. In addition to the D208S mutation with which it was isolated, the R85H mutation also restored motility to the D208-E, H, Q, N, and A mutants (Table 2.4). Thus, several nonprotonatable side-chains can function at position 208 of FlhA, provided the R85H mutation is present. The G223V mutation proved less effective as a suppressor, failing to rescue the motility defect in the D208-S, Q, N, or A mutants. The R85H mutation was also tested in combination with the Asp-158 mutation D158N, but motility was not restored.

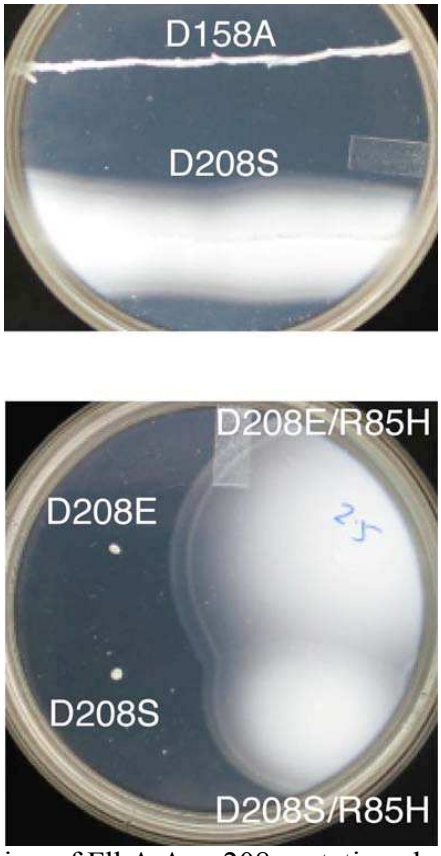

Figure 2.3. Suppression of FlhA Asp-208 mutations by additional mutations in FlhA. *Top:* Cells of the D208N mutant, inoculated in streaks on a soft-agar plate and incubated for 36 h at 37° C. Motile variants are observed migrating out from the streaks. Bottom: Motility of FlhA double mutants with nonprotonatable side-chains at position 208.

Table 2.4. Intragenic suppression of FlhA residue-208 mutants (relative migration rates in soft-agar motility plates)

\ 2nd mutation 1st mutation\	none	R85H	G223V
D208E	0	.8	.1
D208H	0	.8	
D208S	0	.5	0
S208Q	0	.4	
D208N	0	.3	
D208A	0	.2	

Substrate secretion in the FlhA mutants

The motility defects in the FlhA Asp-158 and Asp-208 mutants are presumably due to failures in flagellar export. To assay export more directly, proteins in culture supernatants were precipitated and levels of FliC (flagellin) were estimated on immunoblots (Figure 2.4). Each of the Asp 158 replacements prevented export of FliC, with the exception of the D158E replacement, which also retained motility. All replacements of Asp 208 were defective in FliC export, mirroring the motility defects observed at that position.

Cytoplasmic location of the FliQ and FliR C-termini

The sequence-based topology-prediction methods reached fair consensus for most membrane components of the T3S apparatus (Table 2.2), and some constraints are also available from experiment. FliP contains a cleavable signal sequence (34), locating the N-terminus of the mature protein in the periplasm, followed by four strongly predicted membrane segments that would give the topology shown in Figure 2.1. The topologies of FlhB and FlhA are constrained by the presence of large carboxyl-terminal domains in the cytoplasm. If FlhB contains four TM segments as the majority of the algorithms predict, then the N-terminus of FlhB would also be in the cytoplasm, as should be the C-terminus of FliR given the existence of a functional FliR-FlhB fusion protein (77). As noted above, experiments using fusions to a tandem LacZ/PhoA reporter indicated a quite different topology for the FliR-homolog HrcT, having just a single TM segment and the bulk of the protein, including the C-terminus, in the periplasm (92). We examined FliR topology using individual C-terminal fusions to LacZ or PhoA. The results (Figure 2.5) support a

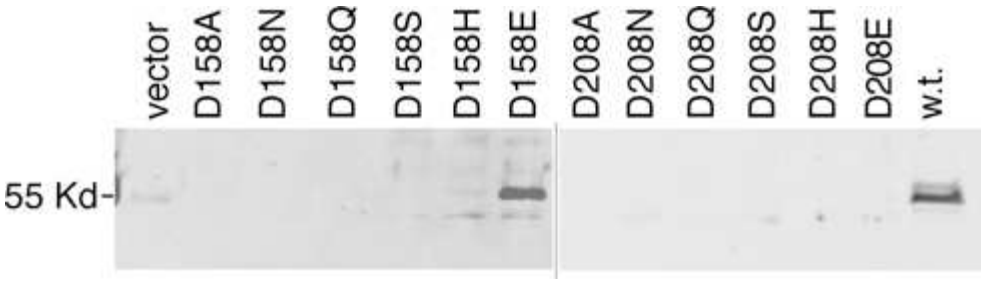

Figure 2.4. Effects of FlhA mutations on export of the flagellar-filament protein FliC. Mutant variants of FlhA were expressed from plasmids in the Δ*flhA* strain, induced with 2.5 μM salicylate. FliC in culture supernatants was precipitated and visualized on anti-FliC immunoblots.

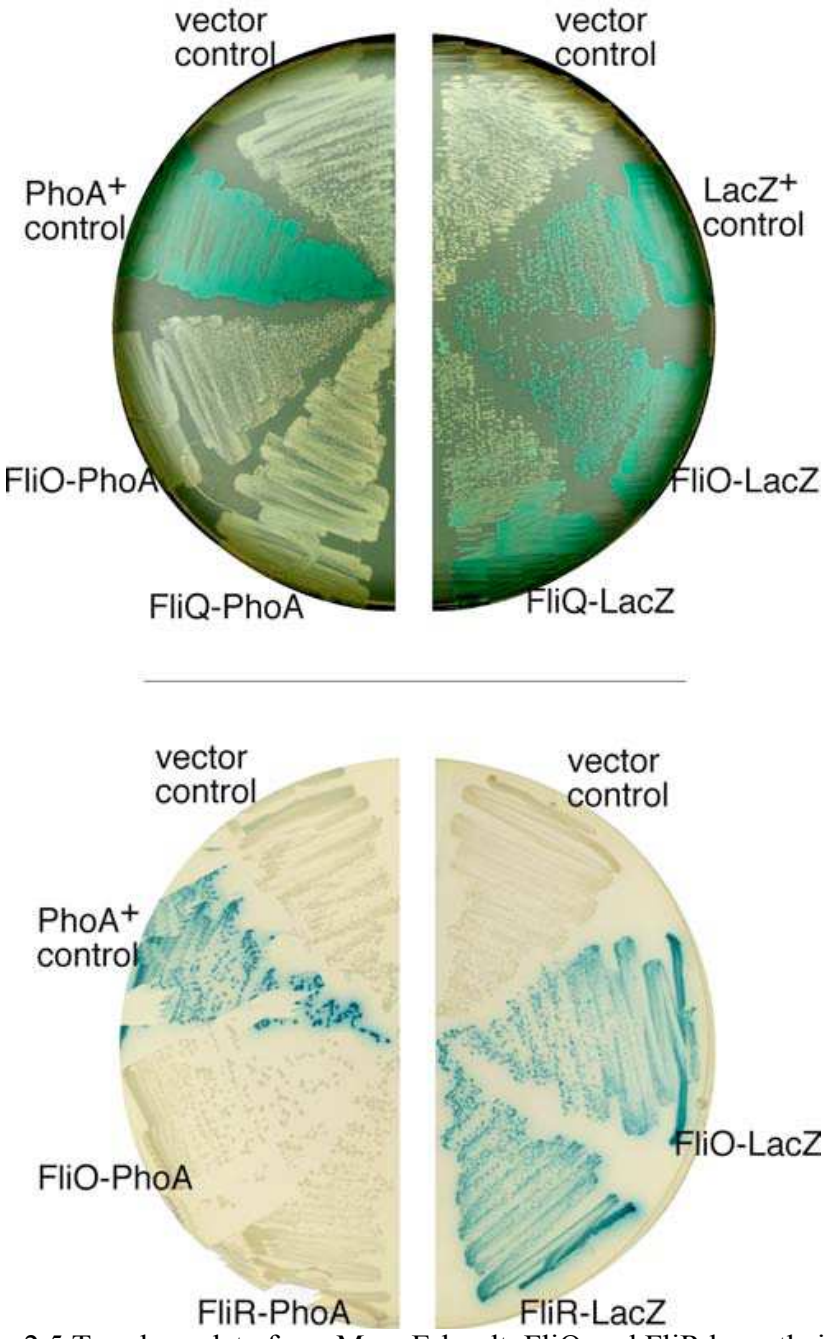

Figure 2.5 Topology data from Marc Erhardt. FliQ and FliR have their C-termini in the cytoplasm. Experiments used C-terminal fusions to PhoA or to LacZ, as indicated. Plates contained X-Pho or X-Gal (see Materials and Methods). The vector control was pTrc99a, the Pho^+ positive control was pTrc99a expressing Flk(1-333)-PhoA, and the $LacZ^+$ positive control was pTrc99a expressing Flk-TM6(LacY)-LacZ. FliO, which has been shown to have its C-terminus in the cytoplasm (32), is included as an additional control.

cytoplasmic location for the FliR C-terminus, consistent with the topology shown in Figure 2.1 and in contrast to the conclusions obtained using the tandem-LacZ/PhoA reporter. In the case of FliQ, all methods predicted two TM segments but did not show clear consensus on their orientation (termini in the cytoplasm *vs*. periplasm). We examined FliQ topology experimentally using C-terminal LacZ and PhoA fusions. The results (Figure 2.5) support the FliQ topology given in Figure 2.1, having both termini in the cytoplasm, and again contrast with the conclusions from the tandem-LacZ/PhoA reporter study (92).

Disscussion

Topologie

The fair agreement among various TM-segment prediction algorithms indicate the presence, in most cases, of well-defined hydrophobic segments. Although the experimental study of the *Xanthomonas* Hrc proteins (92) led to very different conclusions regarding the FliQ-homolog HrcS and the FliR-homolog HrcT, these are not likely to reflect real differences between the systems, because the systems show strong homology at the sequence level and the computational predictions for HrcS and HrcT (also reported in (92)) resemble those for FliQ and FliR. The present experiments using more-conventional individual LacZ and PhoA fusions (Figure 2.5) gave results in support of the topologies shown in Figure 2.1, and thus in agreement with the computational predictions. We suggest that the study of the *Xanthomonas* protein topologies may have been compromised by the use of an unusually large reporter construct, together with sizable linking segments that were themselves fairly hydrophobic.

Functionally critical regions in the T3S apparatus

A previous study gave evidence that FliP forms the transmembrane conduit for the exported protein subunits (35), and the membrane segments of FliP exhibit quite strong sequence conservation that would be consistent with such an essential function. Some of the FlhA membrane segments are similarly well conserved. Given the occurrence of critical proton-bonding residues in FlhA, we propose that the conservation of its membrane segments might reflect their involvement in proton conduction. Segments TM-2, TM-3, TM-5, and TM-6 in particular have properties suited to such a channel function. Each contains polar groups (from the side-chains of Ser, Asn, and Thr) that might contribute to the lining of a channel. Segment TM-3 contains an invariant Arg residue near its middle (residue 85, the position that gave rise to a suppressor of Asp-208 mutations), and TM-5 carries the important acidic residue Asp-208 at its inner end.

The present results show that nonprotonatable residues can function in place of Asp 208, provided that certain other mutations are present elsewhere in FlhA. A comparison of FlhA sequences shows, further, that in the *T. pallidum* protein Asp 208 is replaced by Asn and the otherwise conserved Arg residue at position 85 is replaced by Gly, thus presenting a phylogenetic variant with alterations in the same positions as the D208S/R85H pair isolated as a suppressor. Because there is no strict requirement for a protonatable side-chain at position 208, we suggest that Asp 208 is unlikely to be a site where proton binding is coupled to the export process. This residue is nevertheless important for function, and on the basis of its location we hypothesize that Asp 208 might facilitate proton movement in the lower part of the channel. In this model, the

suppressing mutations in residues Arg 85 and Gly 223 would act by altering some features of the channel, possibly enlarging it, so that the requirement for an acidic residue at the lower end is relaxed. The role of Arg 94, at the outer end of TM-3, is less clear. It might serve a structural role, stabilizing the conformation of the protein or maintaining needed interactions with other components, or it might interact with other titratable groups in the vicinity to form a site that speeds the acquisition of protons and facilitates their delivery into the channel.

Hypothesis for the transport mechanism

The most strictly conserved region of FlhA is a cytoplasmic domain of about 60 residues between TM-segments 4 and 5. This domain contains Asp 158, the proton-binding residue found here to be most critical for function. The present results point to a strict requirement for an acidic residue (which can be either Asp or Glu) at this position. No other critical proton-binding residues were found in this region of FlhA, either in the inner parts of the TM segments or the cytoplasmic domains. We propose, therefore, that Asp-158 is the site where proton binding is harnessed to the processes of substrate transport. A simple transport mechanism based on this proposal, involving proton-regulated conformational changes in the FlhA cytoplasmic domains, is illustrated in Figure 2.6.

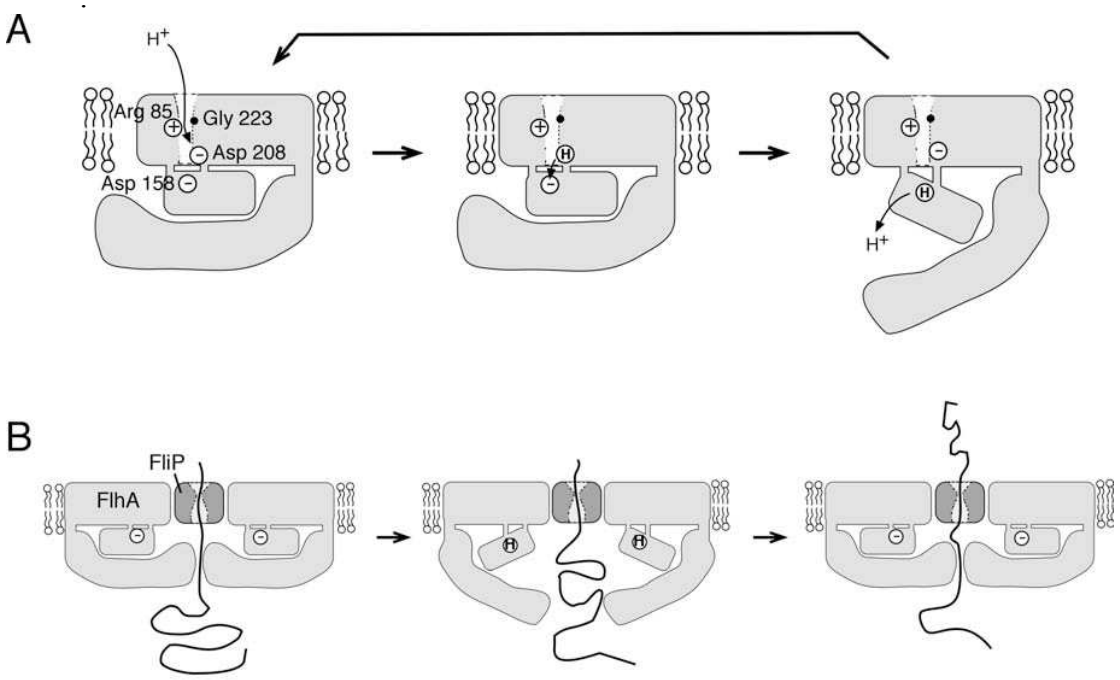

Figure 2.6. Hypothesis for the function of FlhA in flagellar export. (**A**) Proposed role of conserved residues of FlhA, and an hypothesized conformational change. Asp 208 is near the lower end of the channel where it facilitates proton delivery to Asp 158 in the highly conserved small cytoplasmic domain. Protonation of Asp 158 triggers movement or conformational change in the domain, which in turn actuates movement within the larger C-terminal cytoplasmic domain. Arg 85 and Gly 223 are near the middle part of the channel. The suppression effects (Figure 2.3) would be explained if mutations at these positions alter the channel so that a proton-binding residue is no longer required at the inner end. (**B**) A mechanism for substrate export based on movements in the FlhA cytoplasmic domains. The apparatus is assumed to contain multiple FlhA subunits (two are shown but more are possible). FliP is pictured at the center of the apparatus where it forms the conduit for the exported substrate (35). The FlhA cytoplasmic domain engages the substrate, and proton-actuated movements in the domain induce substrate movement through the central conduit.

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